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Transcriptional regulators transforming growth factor-β1 and estrogen-related receptor-α identified as putative mediators of calf rumen epithelial tissue development and function during weaning[1]

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ABSTRACT

Molecular mechanisms regulating rumen epithelial development remain largely unknown. To identify gene networks and regulatory factors controlling rumen development, Holstein bull calves (n = 18) were fed milk replacer only (MRO) until 42 d of age. Three calves each were euthanized at 14 and 42 d of age for tissue collection to represent preweaning, and the remaining calves were provided diets of either milk replacer + orchard grass hay (MH; n = 6) to initiate weaning without development of rumen papillae, or milk replacer + calf starter (MG; n = 6) to initiate weaning and development of rumen papillae. At 56 and 70 d of age, 3 calves from the MH and MG groups were euthanized for collection of rumen epithelium. Total RNA and protein were extracted for microarray analysis and to validate detected changes in selected protein expression, respectively. As expected, calves fed MRO had no rumen papillae and development of papillae was greater in MG versus MH calves. Differentially expressed genes between the MRO diet at d 42 (preweaning) versus the MG or MH diets at d 56 (during weaning) were identified using permutation analysis of differential expression. Expression of 345 and 519 transcripts was uniquely responsive to MG and MH feeding, respectively. Ingenuity Pathway Analysis (Qiagen, Redwood City, CA) indicated that the top-ranked biological function affected by the MG diet was the cell cycle, and TFGB1, FBOX01, and PPARA were identified as key transcriptional regulators of genes responsive to the MG diet and associated with development of rumen papillae. Increased expressions of *TGFB1* mRNA and protein in response to the MG diet were confirmed by subsequent analyses. The top-ranking biological function affected by the MH diet was energy production. Receptors for IGF-1 and insulin, ESRRA, and PPARD were identified by ingenuity pathway analysis as transcriptional regulators of genes responsive to the MH diet. Further analysis of *TGFB1* and *ESRRA* mRNA expression in rumen epithelium obtained from a separate ontogenic study of Holstein calves (n = 26) euthanized every 7 d from birth to 42 d of age showed increases in transcript expression with advancing age, supporting their roles in mediating rumen epithelial development and function during weaning. Additional evaluation of gene expression in the rumen epithelium of adult cows ruminally infused with butyrate also suggested that observed changes in *ESRRA* mRNA expression in developing calf rumen may be mediated by increased butyrate concentration. Our results identify TGFB1 and ESRRA as likely transcriptional regulators of rumen epithelial development and energy metabolism, respectively, and provide targets for modulation of rumen development and function in the growing calf.

Key words: dairy calf, gene expression, rumen development

INTRODUCTION

Proper development and function of the rumen at weaning are critical to the health and productivity of dairy calves (Baldwin et al., 2004; Khan et al., 2011). Recently, gene transcript profiling of the rumen epithelium has been used to gain a better understanding of molecular changes occurring in the ventral sac during weaning (Laarman et al., 2012; Connor et al., 2013), in response to nutritional plane of neonatal dairy calves (Naeem et al., 2012, 2014), and in dietary transitions of adult cattle (Penner et al., 2011; Steele et al., 2011). For instance, mRNA expression patterns of critical transporters affecting rumen pH and nutrient metabolism have been characterized during weaning to improve our understanding of the molecular changes that occur dur-

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ing the establishment of rumen fermentation and VFA production. Likewise, putative transcriptional regulators of genes participating in FA metabolism, ketogenesis, and cholesterogenesis, including the peroxisome proliferator-activated receptors (**PPAR**) in developing rumen epithelium have been identified (Laarman et al., 2012; Naeem et al., 2012, 2014; Connor et al., 2013). Steele et al. (2011) used microarray and quantitative PCR approaches to identify molecular pathways involved in FA metabolism and cholesterol homeostasis in rumen epithelium that are associated with SARA and rumen epithelial adaptation to high-concentrate diets in mature dairy cows. Thus, transcriptomics and gene pathway and network analysis can be used effectively to generate testable hypotheses regarding mechanisms controlling rumen development and function during the critical stages of life of a dairy animal and aid in the creation of technologies that improve these processes to enhance dairy production.

Establishment of rumen fermentation and effective absorption of VFA are critical physiological transitions for the neonatal calf; thus, we wished to gain a better understanding of the molecular mechanisms controlling these processes, including the development of rumen papillae and changes in rumen epithelial cell function during weaning. Because it is well established that VFA, particularly butyrate and propionate, have a substantial effect on differentiation of the rumen epithelium and papillary development during weaning (Flatt et al., 1958; Sander et al., 1959; Mentschel et al., 2001), as well as during dietary transitions in adult ruminants (Sakata and Tamate, 1978, 1979), we chose to use dietary treatments to induce differential rumen development in neonatal calves to examine transcriptional changes in the rumen epithelium using a microarray approach. Specifically, it has been demonstrated that diets that have a large proportion of concentrates promote increased density and length of rumen papillae relative to diets predominated by forages (Harrison et al., 1960; Stobo et al., 1966; Žitnan et al., 2003), which primarily increase rumen volume (Warner et al., 1956). Indeed, differential development of the rumen epithelium using diets of milk only versus milk supplemented with hay or grain at weaning has been demonstrated (Heinrichs, 2005). Thus, we wished to use the same rumen development model to compare gene expression patterns in rumen epithelium of preweaned calves fed only conventional milk replacer to the expression patterns of weaning calves supplemented with forage in the form of hay to stimulate only rumen growth, or supplemented with concentrate in the form of grain-based calf starter to stimulate development of rumen papillae.

Using this model, we previously evaluated changes in gene expression in the calf rumen that occurred in common to supplementation of milk replacer in the form of both grain and hay at weaning relative to feeding of milk replacer alone to characterize molecular pathways responsive to weaning, regardless of the type of solid feed provided and the predominant VFA produced during establishment of fermentation (Connor et al., 2013). That study identified transcription factor PPAR-α (**PPARA**) as a likely mediator of observed transcriptional responses to feeding of a solid diet. Therefore, the goal of the present study was to compare expression profiles of genes from the same calf trial that were differentially responsive to grain versus hay feeding at weaning to identify gene networks and transcriptional regulators contributing to the contrasting growth responses of the rumen epithelium induced by these 2 diets. Because high-forage diets in the growing calf result in a larger rumen without development of papillae, and high-concentrate diets stimulate mucosal development and differentiation of rumen papillae, we hypothesized that hay feeding would induce expression of genes and pathways participating in cellular proliferation and growth, whereas grain feeding would induce genes and pathways controlling cellular differentiation. Selected transcriptional regulators identified by Ingenuity Pathway Analysis (**IPA**; Qiagen, Redwood City, CA) as important mediators of molecular changes occurring in response to hay and grain feeding were then evaluated in rumen samples obtained from 2 independent experiments to verify their roles in mediating rumen development and epithelial cell function.

MATERIALS AND METHODS

All procedures involving animals were approved by the Beltsville Area Animal Care and Use Committee Protocol Number 07–025.

Animals and Tissue Collection

A total of 18 Holstein bull calves [41 ± 7 (SD) kg of BW] born between January 24 and May 6 were purchased from a private dairy farm in New Windsor, Maryland, and transported to the Beltsville Agricultural Research Center (Beltsville, MD) by 3 d of age. Calves were provided with colostrum at their first feeding. Once at the Beltsville Agricultural Research Center, calves were housed individually in concrete pens (7.3 m^2) with a rubber mat as a bedding surface within a single environmentally controlled building. Calves were assigned to 1 of 3 dietary treatments (6 calves/treatment) and were euthanized at 2 different time points (n = 3 per time point) within dietary treatment, as described herein. Previous research from our laboratory demonstrated that a sample size of 3 is suf-

ficient to detect differences in gene expression related to organ development in dairy cattle (Connor et al., 2008). Dietary treatments included milk replacer only (**MRO**) to represent preweaned calves; milk replacer + grain-based commercial calf starter (**MG**; Bel Air Calf Starter 22%, Bel Air, MD) to stimulate rumination and development of rumen papillae; or milk replacer + orchard grass hay (**MH**; fed free choice) to stimulate rumination and rumen growth, but no development of rumen papillae. All calves had continuous access to water and were fed milk replacer twice daily (1.9 L per feeding for a total of 3.8 L/d as recommended by the manufacturer; Cornerstone 22:20, Purina Mills, St. Louis, MO; 22.0% CP, 20.0% crude fat, 0.15% crude fiber, 0.75–1.25% Ca, 0.70% P, 66,000 IU/kg of vitamin A, 11,000 IU/kg of vitamin D_3, and 220 IU/kg of vitamin E) until 42 d of age. Thereafter, calves were fed the MH or MG diets as previously described (Connor et al., 2013). Mean (± SD) birth weight of calves was 45 ± 5 and 39 ± 6 kg for d 14 and 42, respectively, for MRO; 44 ± 9 and 34 ± 6 kg for d 56 and 70, respectively, for MH; and 42 ± 7 and 40 ± 5 kg for d 56 and 70, respectively, for MG. Calves were euthanized at 14 and 42 d of age in the MRO group to evaluate rumen development preweaning. Calves in the MH and MG groups were assigned to treatments and euthanized in pairs (1 calf per dietary group) at 56 and 70 d of age to evaluate rumen development during weaning in response to feeding of hay (forage) or grain (concentrate).

Calves were euthanized by captive bolt stunning followed by exsanguination, and the digestive organs were removed immediately. Tissue was collected from the anterior portion of the ventral sac of the rumen beneath the reticulum and below the rumen fluid layer. The rumen epithelium was manually separated from the underlying muscular layer by gloved hands and rinsed in tap water to remove residual feed particles. Samples of rumen epithelial tissue (including developing papillae if present) were fixed in RNAlater (Life Technologies, Grand Island, NY) RNA stabilization solution according to the manufacturer's instructions for RNA extraction. Samples also were snap-frozen in liquid nitrogen without RNAlater treatment for protein extraction. All samples were stored at −80°C until processed. Total RNA [mean (± SD), RNA integrity number of 8.4 ± 0.7] was used for hybridization to a custom oligonucleotide-based bovine whole-genome array (USDA Bovine 60mer 344-k-feature array representing approximately 45,000 unique bovine transcripts; Li et al., 2006) and subsequent validation of changes in gene expression by real-time quantitative reverse transcription-PCR (**qPCR**). Protein was used for ELISA of a protein of interest (transforming growth factor β1; **TGFB1**) based on microarray results.

RNA Extraction and Transcript Profiling

Frozen fixed tissues were homogenized in Trizol Reagent (Invitrogen, Carlsbad, CA) and total RNA was extracted using the RNeasy Midi Kit (Qiagen, Valencia, CA) with on-column DNase digestion. Quality and concentration of RNA was assessed using a 2100 Bioanalyzer and RNA 6000 Nano LabChip kits (Agilent Technologies, Palo Alto, CA) and NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE), respectively. Probe labeling and microarray hybridization were performed as previously described (Connor et al., 2013).

Determination of Differential Gene Expression

Two 2-group comparisons of differential gene expression were made, including MRO at d 42 versus MH at d 56 and MRO at d 42 versus MG at d 56. Differential expression was evaluated by Expression Analysis Inc. (Durham, NC) using permutation analysis of differential expression, as described in Connor et al. (2013; and at http://www.expressionanalysis.com/docs/PADE_Tech_Note.pdf). Lists of differentially expressed transcripts for each 2-group comparison were then created by selecting transcripts with a small false discovery rate (<0.05), and then further reduced by selecting only transcripts with an estimated absolute raw fold change ≥2.0 because a combination of statistical and fold change criteria provides more reproducible results (Shi et al., 2008; McCarthy and Smyth, 2009). Raw estimated fold changes for each gene transcript based on microarray hybridization were calculated as the simple ratio of the geometric means of the signal values for each dietary group being compared. Transcript identity was determined by aligning the specific oligonucleotide probe sequences used on the array to the bovine genome (UMD 3.1; ENSEMBL Genebuild v65.0) using TopHat (Trapnell et al., 2009) with a cutoff of 2 mismatches tolerated per 60 bp of input sequence. Two final gene lists were then generated from the 2-group comparisons: (1) grain-specific (**GRAIN**) genes, in which expression was changed in response to time and the transition from the MRO diet at d 42 to the MG diet at d 56 but that did not overlap with genes changed in response to the transition to the MH diet at d 56; and (2) hay-specific (**HAY**) genes, in which expression was changed in response to time and the transition from the MRO diet at d 42 to the MH diet at d 56 and age but that did not overlap with genes changed in response to the transition to the MG diet at d 56. Although gene changes in response to diet and time are confounded in our developmental model, removal of overlapping genes from the GRAIN and HAY

lists should minimize the number of genes included in the data set that were changed due to the factor of time (or age), as these changes would be expected to occur similarly in both dietary groups. Genes that were commonly affected by transition from the MRO diet at d 42 to either the MG or MH diets at d 56 were the focus of the report by Connor et al. (2013). Note that a limited number of genes in the present work may appear in the previous report as being commonly responsive to both diets because a less stringent criteria for differential expression (false discovery rate <0.05 and 1.5-fold change cut-off) was used in the previous study to capture smaller effects on gene expression related to dietary transition. Validation of differential gene expression detected by microarray hybridization was conducted previously on a subset of 24 genes of interest by qPCR (Connor et al., 2013). Fold changes detected by microarray and qPCR were positively correlated ($r = 0.57$, $P < 0.01$) and differential expression was confirmed by qPCR for 22 genes at $P < 0.05$ and for the remaining 2 genes at $P < 0.10$.

Gene Network Analysis

Gene pathway and network analysis on the GRAIN and HAY lists were performed using IPA Software (www.ingenuity.com; Ingenuity Systems Inc./Qiagen, Redwood City, CA). Genes and their corresponding raw estimated fold changes were uploaded in the software application and each gene was mapped to its corresponding object in the Ingenuity Knowledge Base. These molecules were overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base to form algorithmically generated networks based on their connectivity. Each gene list was evaluated using the Ingenuity Knowledge Base (genes only) reference set and included direct and indirect relationships, as well as endogenous chemicals. A filter summary was used that considered only molecules or relationships where the species equaled mouse, rat, or human. To determine biological functions of networks, network molecules associated with biological functions in the Ingenuity Knowledge Base were analyzed. By default, IPA software uses a right-tailed Fisher's exact test to calculate a *P*-value for the probability that each biological function assigned to that network was due to chance alone. A score was assigned where the score equals the negative log of the *P*-value. Identification of upstream transcriptional regulators was assessed using IPA default transcriptional regular analysis wherein the activation or inhibition of a transcriptional regulator is predicted using an algorithm. Using information stored in the Ingenuity Knowledge Base regarding known target genes of transcriptional regulators and the effects of the transcriptional regulators on target gene expression reported in the scientific literature, the analysis counts the number of known gene targets and their direction of change (upregulation or downregulation) that occur in the experimental data set to identify likely transcriptional regulators. The absolute value of the regulatory z-score, ≥2.0, is considered statistically significant, wherein a positive value indicates activation and a negative value indicates inhibition of the regulatory factor.

qPCR

Three upstream regulators of gene expression identified by IPA as important among the HAY and GRAIN lists were selected for validation using qPCR analysis of RNA obtained from the current study and rumen epithelial RNA obtained from 2 additional independent experiments. One experiment was conducted in adult lactating Holstein cows ruminally infused with butyrate (Baldwin et al., 2012) and a second was conducted in conventionally reared Holstein heifer calves euthanized at 7-d intervals from birth to 42 d of age. Specifically, expression levels of transforming growth factor-β1 (*TGFB1*) and tumor protein p53 (*TP53*) selected from analysis of the GRAIN data set, and estrogen-related receptor α (*ESRRA*) mRNA selected from the HAY data set were quantified using the iCycler iQ Real-time PCR Detection System (Bio-Rad, Hercules, CA) as described by Connor et al. (2013).

The adult cows (n = 4) from the study of Baldwin et al. (2012) were continuously infused via a ruminal cannula with buffered 2.5 *M* butyrate at a rate of 5 L/d and rumen epithelium was harvested by biopsy at d 0 (before infusion), 1, 3, and 7 during butyrate infusion, and at d 1 and 7 after infusion was terminated for RNA extraction and analysis of gene expression. These samples allowed us to examine the specific effects of butyrate, a VFA known to be produced and increase in the rumen during weaning, on target gene expression. The heifer calves were euthanized at birth (n = 4) and at d 7 (n = 4), 14 (n = 4), 21 (n = 3), 28 (n = 4), 35 (n = 3), and 42 (n = 4) of age as part of a separate ontogenic study conducted at Clemson University in accordance with the Clemson University Institutional Animal Care and Use Committee. These samples allowed us to validate the effects of age and conventional weaning on target gene expression obtained from a completely independent study. In the current study, rumen epithelium was collected from the ventral sac below the liquor layer. All calves were fed colostrum at birth then fed Nurture Plus EZ-M milk replacer (Vigortone Ag Products, Brookville, OH; 22% CP, 20% fat, and Bio-Mos; 283.5 g/1.9 L) twice daily.

Calves were provided ad libitum access to water and Carolina Choice texturized calf starter (Newberry Feed and Farm Inc. Newberry, SC) medicated with lasalocid (18% CP, 2.5% crude fat, and 6.5% crude fiber).

Primers sequences for bovine *TGFB1* and were 5′-TGAGCCAGAGGCGGACTACT-3′ (sense) and 5′-TGCCGTATTCCACCATTAGCA-3′ (antisense), as previously reported by Sugawara et al. (2010), and the annealing temperature was 62.6°C. Efficiency of amplification for the 3 *TGFB1* assays ranged from 93 to 106%. Bovine *TP53* primers sequences were designed with primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were 5′-GTCATCTAGCGTCCGACCTC-3′ (sense) and 5′-TGTCTCCTGACTCAGAGGGG-3′ (antisense) and the annealing temperature was 62.6°C. The amplification product was directly sequenced using the GenomeLab GeXP Genetic Analysis System and DTCS Quickstart Kit (Beckman Coulter, Brea, CA) to confirm amplification of the expected target. Efficiency of amplification for the 3 assays ranged from 102 to 104%. Expression of *ESRRA* mRNA was quantified using primers and amplification conditions, as previously reported (Connor et al., 2005). Efficiencies of amplification for the 2 assays were 86 and 93%. Linearity of all qPCR assays was acceptable ($R^2 \geq 0.997$).

Protein Extraction and ELISA

To characterize changes in TGFB1 protein expression in rumen epithelium in response to diet during weaning, ~100 mg of each flash-frozen rumen epithelial sample was homogenized in lysis buffer (20 m*M* Tris pH 7.4, 150 m*M* NaCl, 1% Triton-X100; supplemented with protease-inhibitor cocktail) using the Bullet Blender 24 (Next Advance, Averill Park, NY) for 5 min at speed setting 8. Protein concentrations were determined by the BCA protein assay (Thermo Scientific, Pittsburgh, PA) using known BSA concentrations to fit an 8-point standard curve ranging from 100 to 2,000 μg/mL ($R^2 = 0.986$). In vivo processed TGFB1 protein abundances were determined in triplicate on 20 μg of protein by sandwich ELISA using the TGFB1 E_{max} ImmunoAssay System (Promega, Madison, WI) following the manufacturer's protocol. The intraassay and interassay CV were 14.6 and 21.2%, respectively. Linearity (R^2) of the 7-point TGFB1 standard curve ranging from 15.6 to 1,000 pg/mL was >0.997.

Statistical Analyses

Active TGFB1 protein and *TGFB1*, *TP53*, and *ESRRA* mRNA levels in rumen epithelium of calves in the MRO, MH, and MG groups were compared across diets and time (first versus second measurement within diet) by ANOVA using the GLM procedure in SAS (SAS Institute Inc., Cary, NC). Diet and time were the CLASS variables and the MODEL statement included each mRNA transcript level or TGFB1 protein level as a dependent variable and diet, time, and the interaction of diet by time as the independent effects. Subsequent pairwise comparisons of least squares means across diet or time for each mRNA transcript or TGFB1 protein were performed with SAS using the PDIFF option in the LSMEANS statement for each CLASS variable. To evaluate changes in *TGFB1*, *TP53*, and *ESRRA* mRNA in calves from birth to 42 d of age, the GLM procedure of SAS was used to evaluate the effect of age on transcript abundance. Pairwise comparisons of least squares means across age groups were performed using the PDIFF option in the LSMEANS statement. The REG procedure of SAS was used to evaluate the cubic relationships between *TGFB1* or *ESRRA* mRNA expression and calf age. The effect of butyrate infusion on *TGFB1* and *ESRRA* mRNA expression over time in adult cows was analyzed by repeated measures ANOVA using the MIXED procedure of SAS. Time and cow were the CLASS variables and cow was treated as a random effect. Time was considered repeated by cow. Differences between least squares means at each time point were further separated by the ESTIMATE option of SAS. A P-value <0.05 was used to indicate significance for all statistical inferences.

RESULTS

Dietary Effects on Rumen Development

The details of rumen epithelial development are described in Connor et al. (2013). As expected, no development of rumen papillae was observed among tissues collected from the MRO group at 42 d of age. Rumen epithelium collected from calves fed the MH diet exhibited very little development at d 56 of age, whereas development of rumen papillae was apparent in samples collected from calves fed the MG diets at d 56 of age. Calves gained an average (± SD) of 0.2 (±0.2), 0.4 (±0.1), and 0.4 (±0.1) kg/d of BW in the MRO, MH, and MG groups, respectively.

Differential Gene Expression by Microarray Analysis

The microarray data are available as accession number GSE18382 in the Gene Expression Omnibus repository at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/). A total of 345 gene transcripts (Supplementary Table S1; http://dx.doi.org/10.3168/jds.2013-7471) were assigned to the

Table 1. Genes in calf rumen epithelium that are downstream gene targets of transforming growth factor-β1 (*TGFB1*) and were affected by age and weaning to a grain-based diet as identified by ingenuity pathway analysis of 345 differentially expressed genes[1]

Gene symbol	Gene name	Ensemble identification	Prediction[2]	Fold change[3]
ALOX5	Arachidonate 5-lipoxygenase	ENSBTAG00000020319	Activated	2.1
ANKRD1	Ankyrin repeat domain 1	ENSBTAG00000011734	Activated	3.0
BIRC5	Baculoviral IAP repeat containing 5	ENSBTAG00000013573	Affected	2.7
BUB1	Budding uninhibited by benzimidazoles 1 homolog (yeast)	ENSBTAG00000021181	Affected	2.7
CCNA2	Cyclin A2	ENSBTAG00000004943	Inhibited	2.3
CCNB2	Cyclin B2	ENSBTAG00000005269	Affected	3.6
CDK1	Cyclin-dependent kinase 1	ENSBTAG00000010109	Affected	2.1
ESPL1	Extra spindle pole bodies homolog 1 (*Saccharomyces cerevisiae*)	ENSBTAG00000008934	Affected	2.2
GUSB	Glucuronidase, β	ENSBTAG00000000704	Inhibited	2.2
HEY1	Hairy/enhancer-of-split related with YRPW motif 1	ENSBTAG00000015717	Inhibited	−3.3
HLA-DQB1	MHC class II antigen	ENSBTAG00000019588	Inhibited	2.5
IGF2	Insulin-like growth factor 2	ENSBTAG00000013066	Activated	−2.5
MYBL1	V-myb myeloblastosis viral oncogene homolog (avian)-like 1	ENSBTAG00000011981	Affected	2.4
NCAPG	Non-SMC condensin I complex, subunit G	ENSBTAG00000021582	Affected	2.2
NDC80	NDC80 homolog, kinetochore complex component (*S. cerevisiae*)	ENSBTAG00000021673	Affected	2.5
NOS2	Nitric oxide synthase 2, inducible	ENSBTAG00000006894	Inhibited	2.7
PDGFA	Platelet-derived growth factor α polypeptide	ENSBTAG00000014541	Inhibited	−2.9
PMEPA1	Prostate transmembrane protein, androgen induced 1	ENSBTAG00000031524	Activated	2.2
POLE2	Polymerase (DNA directed), epsilon 2 (p59 subunit)	ENSBTAG00000004931	Affected	2.2
PPARG	Peroxisome proliferator-activated receptor gamma	ENSBTAG00000001333	Affected	2.7
SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	ENSBTAG00000014835	Activated	2.2
TGM2	Transglutaminase 2	ENSBTAG00000016208	Activated	2.3
VIM	Vimentin	ENSBTAG00000018463	Activated	2.7

[1]Genes were differentially expressed in rumen epithelium of calves fed a diet of milk replacer only until 42 d of age then supplemented with commercial calf starter (grain) until 56 d of age (MG) relative to calves fed milk replacer only until 42 d of age (MRO).

[2]Predicted state of *TGFB1* activity based on direction of change in target gene expression.

[3]Change in target gene expression between calves in the MG group (n = 3) relative to the MRO group (n = 3).

GRAIN list, which represents those genes in which expression was uniquely altered (minimum 2-fold change and non-overlapping with the HAY list) by transition from the MRO diet at d 42 to a grain-based diet (MG) by d 56. A total of 519 gene transcripts (Supplementary Table S2; http://dx.doi.org/10.3168/jds.2013-7471) were assigned to the HAY list, which represents those genes in which expression was uniquely altered (minimum 2-fold change and nonoverlapping with the GRAIN list) by transition from the MRO diet at d 42 to a hay-based diet (MH) by d 56.

Gene Pathway and Network Analysis

A total of 12 networks (Supplementary Table S3; http://dx.doi.org/10.3168/jds.2013-7471) were identified among the GRAIN genes, all of which had a score >10. Four networks had scores above 20 and were composed of at least 19 focus molecules. These 4 networks included genes involved in (1) cell cycle, cellular assembly and organization, DNA replication, recombination, and repair; (2) connective tissue development and function, tissue morphology, carbohydrate metabolism; (3) cell death, cellular function and maintenance, cell cycle; and (4) cell-to-cell signaling and interaction, cell morphology, tissue morphology. Among the top upstream transcriptional regulators of these genes were TGFB1 (regulation z-score = 0.4; $P < 7.0E-09$), PPARA (regulation z-score = 0.7; $P < 4.4E-06$), forkhead box protein O1 (**FOXO1**; regulation z-score = 1.9; $P < 1.8E-05$), and TP53 (regulation z-score = −1.7; $P < 3.1E-05$). Cell cycle was the most significant biological function associated with these genes (P-value range = 1.5E-09 to 0.02). Table 1 summarizes the downstream targets of TGFB1 as assessed by IPA that were differentially expressed in calf rumen epithelium in response to the MG diet at 56 d of age.

Genes on the HAY list participated in 18 networks scoring >10, of which 3 scored above 20 and had at least 18 focus molecules (Supplementary Table S4; http://dx.doi.org/10.3168/jds.2013-7471). These networks were those participating in (1) cellular assembly and organization, cellular compromise, hypersensitivity response; (2) cell-to-cell signaling and interaction, nervous system development and function, tissue development; and (3) cell cycle, cell morphology, cellular assembly and organization. The insulin-like growth factor 1 receptor (**IGF1R**; regulation z-score = 1.7; $P < 0.003$), insulin receptor (**INSR**; regulation z-score = 2.0; $P < 0.02$), PPAR-δ (**PPARD**; regulation z-score = 0.3; $P < 0.01$), and **ESRRA** (regulation z-score = 1.6; $P < 0.03$) were identified among the upstream

transcriptional regulators of the HAY genes. The top biological function associated with these genes was energy production (P-value range = 2.6E-05 to 0.04), wherein multiple genes function in FA oxidation and regulation of energy metabolism (Table 2).

TGFB1, TP53, and ESRRA Expression

Evaluation of *TGFB1* mRNA expression among the MRO, MG, and MH groups at each time of sacrifice showed no diet by time interaction ($P \leq 0.86$) and no effect of time ($P \leq 0.37$) on *TGFB1* mRNA expression. A difference ($P \leq 0.02$) in *TGFB1* transcript abundance among the dietary groups was detected (Figure 1A), wherein gene expression was upregulated in rumen epithelium of calves in the MG group compared with those in the MRO ($P < 0.05$) and MH ($P < 0.01$) groups by at least 1.3 fold. In addition, for the active TGFB1 protein, no interaction ($P \leq 0.94$) between diet and time and no effect of time ($P \leq 0.93$) on active TGFB1 protein abundances was observed; however, active TGFB1 protein in rumen epithelium differed ($P < 0.002$) among dietary groups in that active TGFB1 was over 20-fold greater ($P < 0.002$) in the MG group relative to the MRO and MH groups (Figure 1B). Furthermore, expression of *TGFB1* mRNA in the separate study of calves from birth to 42 d of age increased ($P < 0.002$) with age, wherein mean expression was increased 3.5 fold at 42 d of age relative to birth (Figure 2A). The best linear fit for the relationship between *TGFB1* mRNA and age was cubic ($R^2 = 0.62$; $P < 0.0001$). The inflection point in *TFGB1* mRNA expression occurred around 21 d of age. Among midlactation adult cows ruminally infused with butyrate from a second additional study, no difference ($P \leq 0.18$) was detected in *TGFB1* mRNA expression over time relative to ruminal butyrate infusion (Figure 2B). Mean *TGFB1* transcript abundance during the period of butyrate infusion was less ($P < 0.02$) than the mean abundance during periods with no butyrate infusion.

Similar to *TGFB1* mRNA, among the MRO, MH, and MG groups, no diet by time interaction ($P \leq 0.43$) and no effect of time ($P < 0.50$) was observed on *TP53* mRNA levels in rumen epithelium. However, *TP53* mRNA was affected ($P \leq 0.05$) by dietary group (Figure 3A). Specifically, *TP53* mRNA expression level in rumen epithelium of calves in the MH group was 1.4-fold lower ($P < 0.02$) than that of calves in the MRO group. Expression of *TP53* mRNA in the MG group did not differ ($P < 0.17$) from the MRO and MH groups. Among the calves from the separate study, no effect ($P \leq 0.90$) of age on *TP53* mRNA expression was noted between birth and 42 d of age (Figure 3B). Likewise, in adult lactating cows ruminally infused with butyrate from the second independent study, no effect of time ($P \leq 0.40$) was observed relative to butyrate infusion on *TP53* mRNA expression in rumen epithelium (Figure 3C).

Microarray results indicated that expression of *ESRRA* mRNA was 2.7-fold greater in the MG group at 56 d of age relative to the MRO group at 42 d of age (Table 2). In the independent study of calves, expression of *ESSRA* mRNA in rumen epithelium increased ($P < 0.0001$) with age, with mean expression 5.0-fold greater at 42 d of age compared with that at birth (Figure 4A). As with *TGFB1* mRNA, a cubic relationship ($R^2 = 0.72$; $P < 0.0001$) was noted between *ESSRA* mRNA and calf age, and the inflection point occurred at about 21 d of age. In the second independent study of adult lactating cows, *ESSRA* mRNA expression in rumen epithelium changed with time relative to butyrate infusion ($P < 0.0002$). During d 1 to 7 of butyrate infusion, *ESSRA* mRNA expression steadily increased ($P < 0.02$), then immediately declined ($P < 0.02$) within 1 d of cessation of butyrate infusion (Figure 4B).

DISCUSSION

Full differentiation and development of the rumen papillae in the growing calf are required for complete and efficient digestion of forage-based diets of the mature animal. Although it is well established that diet and type of VFA produced have a profound effect on the course of rumen mucosal development in the young ruminant, little is known about the molecular mechanisms regulating this critical process. In the mature animal, Penner et al. (2011) reviewed multiple molecular pathways in the rumen epithelium responsive to increased feeding of concentrate diets, including increased expression of growth factors (e.g., IGF-1 and EGF) and their receptors mediating increased cellular proliferation, increased mRNA expression of genes encoding ketogenic enzymes, decreased expression of cholesterol synthesis genes, and increased expression of genes involved in maintaining barrier function and regulation of the inflammatory response. Similar molecular pathways may regulate developmental changes of the calf rumen during the transition from prerumination to rumination in response to consumption of solid feed, particularly high-concentrate calf starter. For instance, similar to observations in rumen epithelium of adult dairy cows fed a high-grain diet versus a high-forage diet (Steele et al., 2011), mRNA expression of a key gene in cholesterol synthesis, 3-hydroxy-3-methylglutaryl-CoA synthase 1 (*HMGCS1*), was reduced by over 2-fold in rumen epithelium of calves by feeding calf starter at weaning relative to hay feeding (Laarman et al., 2012). Likewise, *HMGCS1* mRNA expres-

Table 2. Genes in calf rumen epithelium that function in energy production and were affected by age and weaning to a hay-based diet as identified by ingenuity pathway analysis of 519 differentially expressed genes[1]

Gene symbol	Ensemble gene identification	Gene name	Function	Fold change[2]
ABHD5	ENSBTAG00000008416	1-Acylglycerol-3-phosphate O-acyltransferase	Lipid metabolism and keratinocyte differentiation	2.3
ACADM	ENSBTAG00000024240	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	Fatty acid β-oxidation of acyl chains of 4 to 16	2.1
AK2	ENSBTAG00000017605	Adenylate kinase 2, mitochondrial	Energy metabolism	2.5
ATP5H	ENSBTAG00000021227	ATP synthase subunit d, mitochondrial	Part of mitochondrial complex F0	2.5
ATP5J2	ENSBTAG00000002094	ATP synthase subunit f, mitochondrial	Part of mitochondrial complex F0	2.2
ECI1	ENSBTAG00000009965	3,2-Trans-enoyl-CoA isomerase, mitochondrial	Fatty acid β-oxidation	3.4
ECI2	ENSBTAG00000015178	Peroxisomal 3,2-trans-enoyl-CoA isomerase	Fatty acid β-oxidation	2.3
ESRRA	ENSBTAG00000008645	Estrogen-related receptor α	Increase transcription of genes involved in energy metabolism	2.7
GCDH	ENSBTAG00000016211	Glutaryl-CoA dehydrogenase, mitochondrial	Metabolism of tryptophan	−2.2
GUK1	ENSBTAG00000014775	Guanylate kinase	Kinase activity	2.0
HIF1A	ENSBTAG00000020935	Hypoxia-inducible factor 1-α	Metabolic adaptation to hypoxia to increase O_2 delivery	3.4
HMOX1	ENSBTAG00000015582	Heme oxygenase 1	Heme catabolism	3.5
HPGD	ENSBTAG00000025942	15-Hydroxyprostaglandin dehydrogenase	Metabolism of prostaglandins	2.5
HSD17B10	ENSBTAG00000017779	3-Hydroxyacyl-CoA dehydrogenase type-2	Oxidation of fatty acids, alcohols, and steroids	2.0
MC4R	ENSBTAG00000019676	Melanocortin receptor 4	Energy homeostasis	5.7
NR4A2	ENSBTAG00000003650	Nuclear receptor subfamily 4 group A member 2	Transcriptional regulator	−6.0
NSF	ENSBTAG00000008442	N-ethylmaleimide-sensitive factor	Transport in golgi	2.2
PHYH	ENSBTAG00000007700	Phytanoyl-CoA dioxygenase, peroxisomal	Fatty acid α-oxidation	3.6
PRKAG2	ENSBTAG00000002917	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit	Regulation of cellular energy metabolism	−2.7

[1]Genes were differentially expressed in rumen epithelium of calves fed a diet of milk replacer only until 42 d of age then supplemented with orchard grass hay until 56 d of age (MH) relative to calves fed milk replacer only until 42 d of age (MRO).

[2]Change in target gene expression between calves in the MH group (n = 3) relative to the MRO group (n = 3).

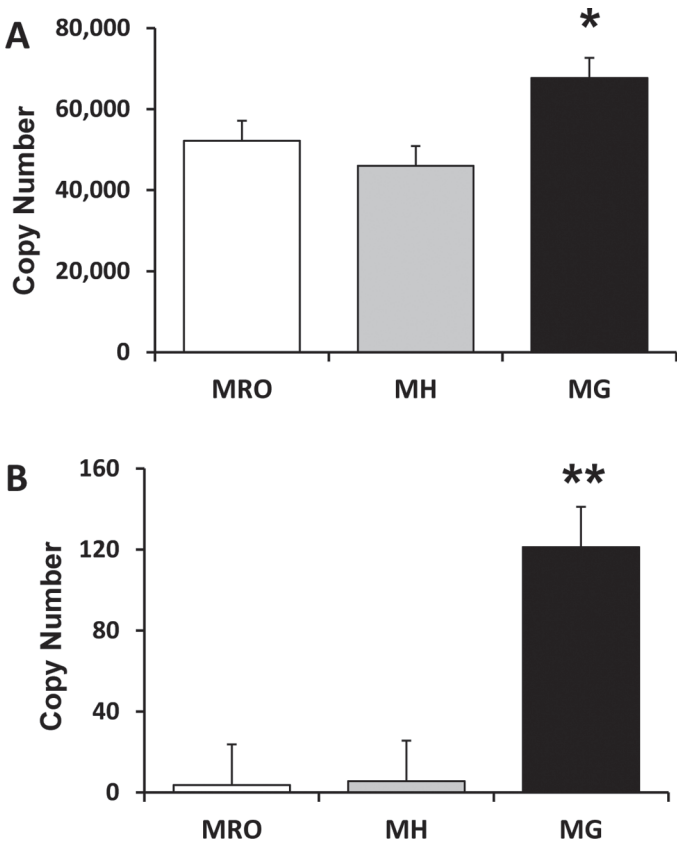

Figure 1. Least squares means (± SE) of expression of (A) transforming growth factor β1 (*TGFB1*) mRNA and (B) naturally processed TGFB1 protein in rumen epithelium of Holstein calves (n = 6 per group) by dietary group. MRO (open bar) = diet of milk replacer only until 42 d of age; MH (gray bar) = diet of milk replacer only through 42 d of age then supplemented with orchard grass hay until 56 d of age; MG (black bar) = diet of milk replacer only through 42 d of age then supplemented with commercial calf starter until 56 d of age. An asterisk (*) represents $P < 0.05$ and two asterisks (**) represents $P < 0.002$; MG vs. MRO or MH.

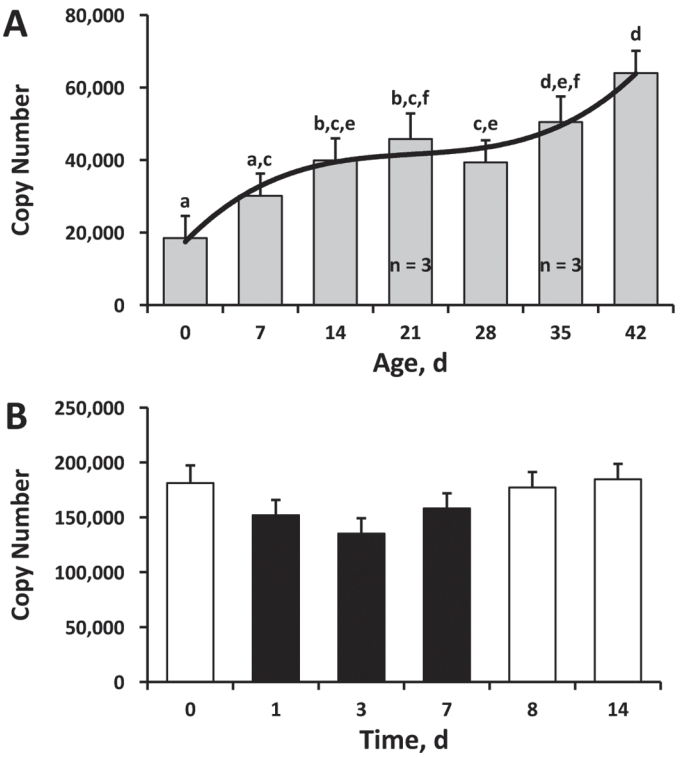

Figure 2. Least squares means (± SE) of expression of transforming growth factor-β1 (*TGFB1*) mRNA in rumen epithelium of (A) Holstein heifer calves from birth to 42 d of age (n = 4 per age unless otherwise noted) and (B) adult midlactation Holstein cows (n = 4) ruminally infused with buffered 2.5 *M* butyrate at a rate of 5 L/d from d 1 to 7 (represented by solid black bars). Rumen epithelium of adult cows was harvested by biopsy at d 0 (before butyrate infusion), 1, 3, and 7 during infusion, and at d 8 and 14 (1 and 7 d, respectively, after butyrate infusion was terminated) for RNA extraction and analysis of *TGFB1* mRNA expression. The cubic regression line shown in A is represented by the equation $y = 2x^3 - 134x^2 + 3{,}003x + 17{,}517$ ($R^2 = 0.62$). Means without a common letter (a–f) differ ($P < 0.05$).

sion was reduced at weaning in rumen epithelium of calves fed a high-protein diet relative to standard protein diet (Naeem et al., 2012). Mechanistically, a more protein-rich diet should increase VFA production, also promoting rumen epithelial cell proliferation and possibly ketogenesis (Penner et al., 2011). Indeed, Naeem et al. (2012) observed increased mRNA expression of the growth-promoting gene *IGF1*, which corresponded to increases in calf age and reticulorumen mass, as well as increased *INSR* mRNA expression in response to a higher protein diet; however, increased expression of selected genes critical to ketogenesis were observed only with increasing age and not in response to diet quality, as might be expected. Overall, because most studies to date characterizing molecular changes in rumen epithelium of developing calves have examined only a limited number of genes of interest, evaluation of global changes in gene expression occurring in the calf rumen during weaning may provide a better understanding of factors controlling mucosal development, epithelial cell proliferation, and cell metabolism during this transitional period of ruminant digestion.

Using the same experimental animals as reported in the present study, we recently evaluated molecular changes in the growing calf rumen epithelium that occurred commonly in both hay and grain feeding during weaning using a microarray approach (Connor et al., 2013). The work identified over 950 gene transcripts affected by weaning that comprised multiple gene networks participating in lipid metabolism, molecular transport, and cellular proliferation, growth, and morphology. Furthermore, many of the changes in gene expression occurring during the period of transition from prerumination to rumination appeared to be mediated by the transcription factor PPARA. This is

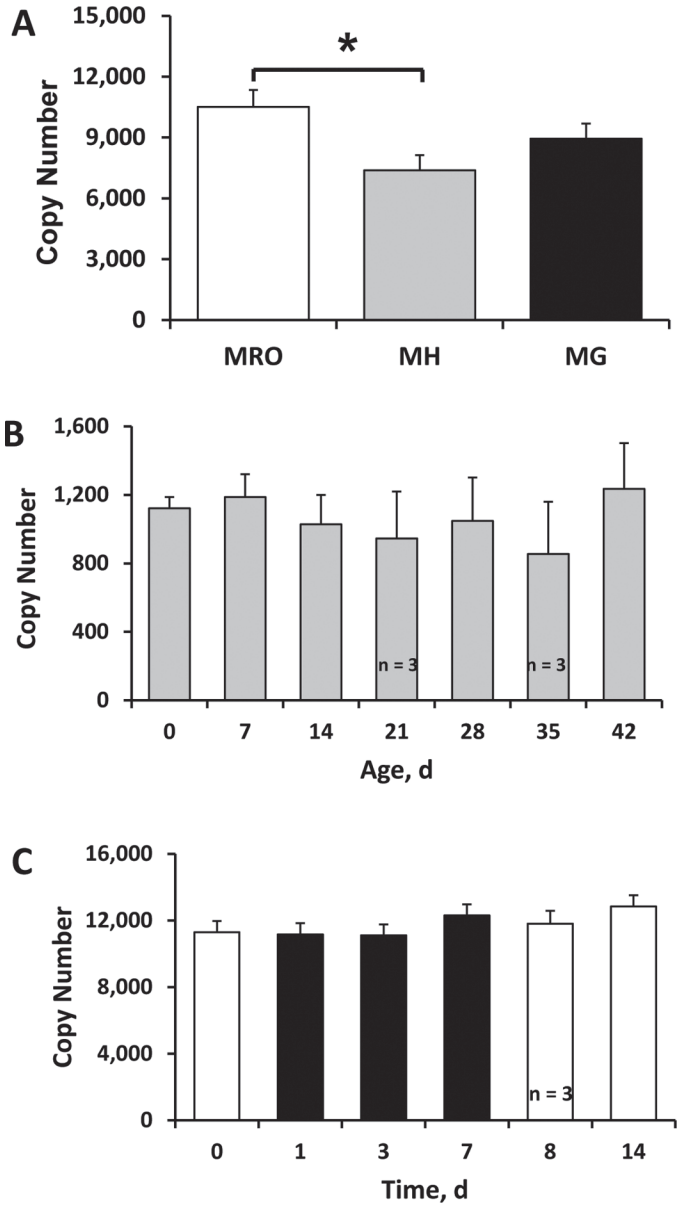

Figure 3. Least squares means (± SE) of expression of tumor protein p53 (*TP53*) mRNA in rumen epithelium of (A) Holstein calves by dietary group [MRO (open bar) = diet of milk replacer only until 42 d of age (n = 5); MH (gray bar) = diet of milk replacer only through 42 d of age then supplemented with orchard grass hay until 56 d of age (n = 6); MG (black bar) = diet of milk replacer only through 42 d of age then supplemented with commercial calf starter until 56 d of age (n = 6; $^*P \leq 0.02$)]; (B) Holstein heifer calves from birth to 42 d of age (n = 4 per age unless otherwise noted); and (C) adult midlactation Holstein cows (n = 4 unless otherwise noted) ruminally infused with buffered 2.5 *M* butyrate at a rate of 5 L/d from d 1 to 7 (represented by solid black bars). Rumen epithelium of adult cows was harvested by biopsy at d 0 (before butyrate infusion), 1, 3, and 7 during infusion, and at d 8 and 14 (1 and 7 d, respectively, after butyrate infusion was terminated) for RNA extraction and analysis of *TP53* mRNA expression.

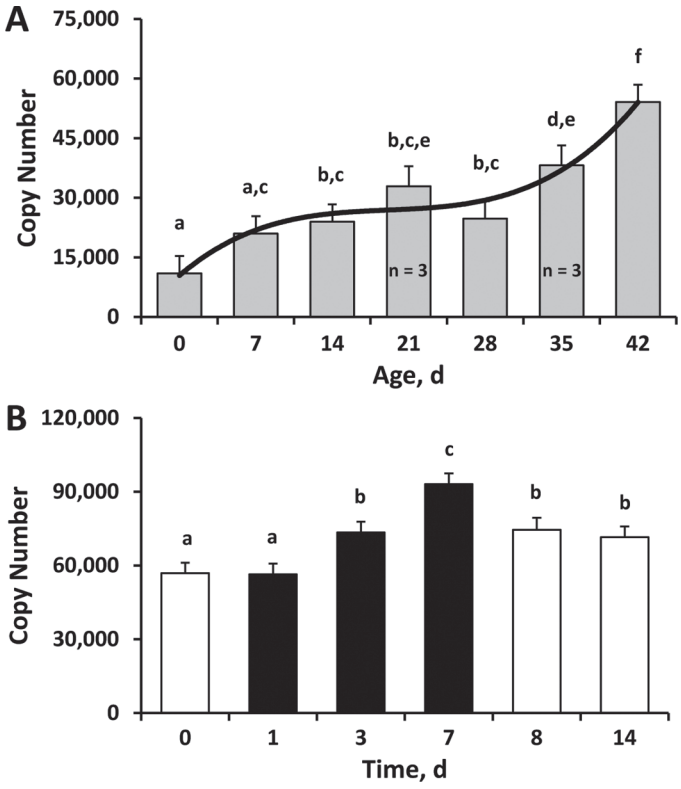

Figure 4. Least squares means (± SE) of expression of estrogen-related receptor α (*ESRRA*) mRNA in rumen epithelium of (A) Holstein heifer calves from birth to 42 d of age (n = 4 per age unless otherwise noted) and (B) adult midlactation Holstein cows (n = 4) ruminally infused with buffered 2.5 *M* butyrate at a rate of 5 L/d from d 1 to 7 (represented by solid black bars). Rumen epithelium of adult cows was harvested by biopsy at d 0 (before butyrate infusion), 1, 3, and 7 during infusion, and at d 8 and 14 (1 and 7 d, respectively, after butyrate infusion was terminated) for RNA extraction and analysis of *ESRRA* mRNA expression. The cubic regression line shown in A is represented by the equation $y = 2x^3 - 117x^2 + 2{,}313x + 10{,}559$ ($R^2 = 0.72$). Means without a common letter (a–f) differ ($P < 0.05$).

consistent with suggested roles of PPARA in cellular differentiation and proliferation, although a role for PPARD in the growth and differentiation of epithelial cells has been demonstrated as well (Burdick et al., 2006). Naeem et al. (2012) reported that the relative abundance of *PPARD* mRNA in the rumen epithelium of weaned calves is considerably greater than that of *PPARA* mRNA, and PPARD may be a more essential factor controlling differentiation of rumen epithelium at weaning. The relative importance of PPARA versus PPARD in rumen epithelial development remains to be determined, but may vary by stage of development as well as by the specific dietary factors or products of cellular metabolism available to serve as PPAR ligands (Barish et al., 2006).

In the present study, we focused on the characterization of changes in gene expression occurring uniquely

to each dietary substrate (i.e., forage vs. concentrate) at weaning, which are associated with specific changes in rumen epithelial growth and development. These expression changes occurred 14 d after the transition from the MRO diet to MG (providing fermentable carbohydrates that produce primarily butyrate during fermentation) versus MH (providing structural carbohydrates that produce primarily acetate during fermentation). As previously reported (Connor et al., 2013), during feeding of MRO, development of rumen papillae did not occur. However, development of rumen papillae occurred in the MH and MG groups by 56 d of age, with longer papillae observed in MG calves. Not surprisingly, corresponding changes in gene expression quantified in rumen epithelium of MG calves identified gene networks functioning primarily in cell replication (cell cycle), tissue morphology and development, and carbohydrate metabolism. Likewise, the cell cycle was the overall biological function identified by IPA as most significantly affected. These gene networks and changes in the cell cycle are consistent with the observed increase in rumen papillary growth and development among these animals at slaughter and when fed a diet rich in fermentable carbohydrates (Stobo et al., 1966). Although production of specific VFA was not assessed in the present study, previous research in Holstein calves fed similar diets indicated that calves supplemented with calf starter at weaning produced 53% more total rumen VFA, consisting of 17% less acetate and 97% more butyrate, compared with calves fed hay at weaning (Laarman et al., 2012). Thus, these molecular changes related to the cell cycle and tissue morphology may be mediated by increased VFA, especially butyrate, among MG calves.

From the differentially expressed genes detected, specifically among the MG calves at weaning relative to preweaned calves on the MRO diet, transcriptional regulators FOXO1, PPARA, TP53, and TGFB1 were identified by IPA. The IPA activation z-scores for FOXO1 and TP53 approached the software's significance thresholds for indicating activation and inactivation, respectively, of these 2 transcriptional regulators. The FOXO1 transcription factor functions through activation of downstream gene targets that suppress the cell cycle, protect the cell from stress, and promote apoptosis (Reagan-Shaw and Ahmad, 2007). Normally, because FOXO1 activity is inhibited by growth factors, such as insulin and IGF-1 (Hay, 2011), we might expect grain feeding also to inhibit FOXO1 activity, promoting rumen epithelial cell growth; however, inactivation of FOXO1 was not observed. Similarly, Naeem et al. (2012) reported increased expression of *FOXO1* mRNA in rumen epithelium of calves fed high-protein milk replacer relative to controls fed standard milk replacer and suggested that FOXO1 serves as a negative feedback signal controlling insulin-mediated cell proliferation during rumen development. Because gene expression was assessed 14 d after transition to the MG diet in the present study, activation of FOXO1 may serve as a negative regulator of cell proliferation that was induced previously by grain feeding. A more detailed characterization of FOXO1 expression and its downstream targets relative to changes in growth factors during rumen development are needed to confirm this hypothesis.

Similar to FOXO1, TP53 is a tumor suppressor that represses cell cycle progression and promotes apoptosis (Levine, 1997). It influences the activity of IGF-1 through transcriptional activation of IGF-binding protein 3, which inhibits IGF-1-mediated effects on cell proliferation (Levine, 1997). Changes in expression of downstream gene targets of TP53 during weaning to the MG diet in the present study suggested that TP53 may be inactivated in response to grain feeding and be an important regulator of epithelial cell proliferation in the calf rumen during its development. In a similar study, Naeem et al. (2014) also reported activation of p53 signaling pathways in rumen epithelium of high-protein-fed calves in response to age and weaning, and inhibition of p53 signaling pathways in high-protein-fed versus control calves preweaning.

Despite no detected differences in *TP53* mRNA expression between preweaned calves and calves in the MG group as assessed by qPCR in the present study, a significant reduction in expression of *TP53* mRNA was detected in the calves of the MH group compared with the MRO group. A reduction in *TP53* mRNA may promote cell proliferation in rumen epithelium of hay-fed calves if reduced transcript abundance corresponds to lower TP53 protein activity in these animals. Although expression of total or active TP53 protein was not evaluated in the current work, we did examine its mRNA expression in the rumen epithelium of conventionally reared dairy calves from birth to 42 d of age obtained from an independent serial slaughter study for further evidence of its role in rumen growth. We found that *TP53* mRNA expression did not change in the rumen epithelium during this earlier stage of development in which some degree of papillary growth had occurred. Furthermore, we evaluated expression of *TP53* mRNA in the rumen epithelium obtained from adult lactating cows infused with butyrate and found no apparent effects of butyrate on *TP53* transcript abundance. The latter finding differs from work in cultured mouse fibroblasts in which butyrate treatment induced *TP53* gene expression and cell cycle arrest (Toscani et al., 1988). Our combined results of *TP53* mRNA expression in rumen of developing calves and adult cows do not support

a prominent role of TP53 in the regulation of rumen epithelial cell proliferation during mucosal development and papillary growth, as was suggested by our initial microarray results.

On the contrary, further evaluation of *TGFB1* mRNA and TGFB1 protein expression among the MRO, MH, and MG groups, as well as *TGFB1* mRNA expression in calves throughout development obtained from the separate ontogeny study, provided supporting evidence of activation of this growth factor during rumen development and a distinct role of TGFB1 in rumen papillary differentiation during grain feeding. Similarly, an increase in TGF-β signaling pathways was reported with age and weaning among dairy calves fed a high-protein diet (Naeem et al., 2014). In an earlier report, butyrate treatment of a human colonic cancer cell line induced *TGFB1* mRNA expression within 24 h of cell treatment (Barnard and Warwick, 1993), suggesting that the effects of grain feeding on *TGFB1* mRNA may be mediated by increased butyrate production. Somewhat unexpectedly, however, no detectable change in overall *TGFB1* mRNA expression was observed over time in the independent study of rumen epithelium obtained from adult cows ruminally infused with butyrate. Furthermore, mean *TGFB1* mRNA expression in rumen epithelium was significantly reduced in the adult cows during the period of butyrate infusion versus combined periods of no infusion.

The discrepancy found in *TGFB1* mRNA expression responses between growing versus adult cows may be due, in part, to differences in the functions of TGFB1 in calf versus adult rumen epithelium. For instance, TGFB1 inhibits cell proliferation, enhances apoptosis, and promotes cellular differentiation (Moustakas et al., 2002; Kubiczkova et al., 2012). In the adult rumen, differentiation of papillae has already occurred; thus, reductions in *TGFB1* mRNA induced by increased ruminal butyrate in the adult animal may relate more to promoting proliferation and elongation of papillae, as opposed to initial differentiation of rumen epithelial cells. Indeed, Goodlad (1981) reported that the mitotic index of the rumen epithelium of mature sheep increases in response to the transition from a high-forage diet to a diet high in concentrates known to produce more butyrate during fermentation. Likewise, Sakata and Tamate (1978) demonstrated that rapid ruminal infusion of butyrate in adult sheep causes a temporary increase in the mitotic index of the epithelial cells of the rumen papillae.

Within this context, a decrease in TGFB1 expression in response to butyrate infusion in a mature ruminant is anticipated to permit cell proliferation and growth of rumen papillae. This is consistent with observed reductions in mean *TGFB1* mRNA expression levels in adult cows during the 7-d butyrate infustion period versus the mean baseline and postinfusion levels. Therefore, our results indicate that TGFB1 may mediate cell proliferation and papillary growth in the rumen epithelium of the adult cow, whereas this growth factor may function to suppress cell proliferation and facilitate rumen epithelial cell differentiation in the growing calf. Further characterization of TGFB1 expression and distribution within the extracellular matrix and among the layers of the rumen epithelium during proliferation and differentiation are needed to gain a better understanding of its function in the ruminal mucosa related to these physiological processes.

Among the MH calves, the most significantly affected gene network was one that functions primarily in cellular assembly and organization, particularly related to cellular compromise. This network included changes in the expression of genes that may reflect the establishment of rumen microbial populations and associated anaerobic fermentation in the rumen during hay feeding, based on the roles of members of this gene network, including inflammation and the immune response [e.g., C-C motif chemokine 2 (*CCL2*), cluster of differentiation 163 (*CD163*), and lymphocyte transmembrane adaptor 1 (*LAX1*)], response to hypoxia [e.g., hypoxia inducible factor 1α (*HIF1A*)], and response to bacterial lipopolysaccharide [e.g., lymphocyte antigen 96 (*LY96*)]. In further support of this, activation of gene transcripts participating in immune function were also reported in rumen epithelium of dairy calves fed a high-protein diet postweaning versus preweaning (Naeem et al., 2014).

Additional gene networks responsive to hay feeding during weaning included those involved in tissue development and the cell cycle. Of interest, similar or overlapping functional activities were identified among the predominant networks of the grain-fed calves, but these functions involved the participation of unique sets of genes under the control of distinctly different transcriptional regulators. The reason for these differences is not known, but likely relates to the differences in the molar ratios of the end products of rumen microbial digestion of each diet [i.e., predominantly acetate from hay versus mostly butyrate from grain (Laarman et al., 2012)] and their direct and indirect effects on transcriptional regulators.

Energy production was identified by IPA software as the most significant biological function affected by weaning to the MH diet based on changes in expression of 19 genes participating predominantly in FA oxidation and the metabolism of lipids and energy. Of note, 12 of the 19 genes are targets of PPAR or interact with PPAR, providing a mechanism for regulation of their transcriptional activity by long-chain FA, NEFA, and,

possibly, glucose (Bionaz et al., 2013) from the blood. Thus, PPAR may be involved in mediating energy metabolism within the rumen epithelium to support cell growth in hay-fed animals and is described in greater detail further in the discussion of transcriptional regulators identified among genes responding to the MH diet.

Relative to the MRO diet, the MH diet should increase rumen VFA production, primarily including acetate, followed by propionate and butyrate (Laarman et al., 2012). These VFA pass into the rumen epithelium, but negligible amounts of acetate and propionate appear to be metabolized by the epithelial cells, whereas most butyrate that may be produced is metabolized by the epithelium and converted to BHBA (Kristensen and Harmon, 2004). Thus, as the rumen epithelium of the MH calves transitions from the carbohydrate-rich MRO diet to one predominated by acetate production, the epithelial cells become more dependent on energy sources from the blood to support cell maintenance and growth. Increased expression of mitochondrial medium-chain specific acyl-CoA dehydrogenase (*ACADM*), which targets metabolism of acyl-CoA chains ranging from C4 to C16, and enoyl-CoA isomerases (*EC1* and *EC2*) involved in β-oxidation of unsaturated FA, in the rumen of calves transitioned to the MH diet may reflect this change in available energy substrates for metabolism.

Transcriptional regulators IGF1R, INSR, ESRRA, and PPARD were identified by IPA as being important mediators of gene expression changes observed in the rumen epithelium in response to the MH diet at weaning. The activation z-scores of the first 3 transcriptional regulators suggested activation of these regulators. Both IGF-1 and insulin have been implicated in mediating the stimulatory effects of VFA on rumen epithelial cell proliferation and growth (Sakata et al., 1980; Neogrády et al., 1989; Baldwin, 1999). Therefore, identification of their receptors in mediating changes in gene expression during rumen development in response to hay feeding at weaning further supports the roles of IGF-1 and insulin in epithelial cell growth. Additionally, Shen et al. (2004) reported that increased growth and development of rumen papillae of juvenile goats was positively related to circulating concentrations of IGF-1 and protein expression of IGF1R in rumen epithelium, suggesting a stimulatory effect of IGF-1 on proliferation of rumen epithelium. Furthermore, Laarman et al. (2012) reported that both plasma IGF-1 and insulin increase in calves between 6 and 8 wk of age, just before weaning, which corresponds to the period in which the rate of increase in rumen weight exceeds the rate of increase in BW in growing ruminants (Baldwin and Jesse, 1992). Last, Žitňan et al. (2005) reported that plasma IGF-1 and insulin concentrations of 42-d-old calves at slaughter were correlated with the length of their rumen papillae and the molar proportions of acetate and propionate in rumen fluid. Thus, our results provide additional evidence supporting the roles of IGF-1 and insulin in rumen epithelial growth and development and suggest that activation of their receptors may be particularly important in mediating the effects of VFA produced during hay feeding relative to grain feeding at weaning.

One of the most interesting findings was the identification of ESRRA as a transcriptional regulator in the calf rumen epithelium in response to hay feeding during weaning. Estrogen-related receptor-α is an orphan nuclear receptor critical for mitochondrial biogenesis and is expressed in tissues that participate in FA β-oxidation and have high energy demands (Giguère, 2008). The ESRRA is known to be expressed in intestinal epithelium of humans and mice and is essential for regulating genes responsible for oxidative phosphorylation and lipid absorption (Carrier et al., 2004), as well as maintenance of energy balance (Ranhotra, 2009). To date, no study has reported on the expression of *ESRRA* mRNA in rumen tissue. In the current study, hay feeding at weaning appeared to activate ESRRA-mediated gene targets. In addition, we demonstrated that expression of *ESRRA* mRNA increased in calf rumen epithelium from birth to 42 d of age and is stimulated by butyrate based on its evaluation in tissues obtained from 2 independent studies. These findings indicate that ESRRA may play a central role in rumen function and energy metabolism of the developing rumen.

Finally, PPARD was identified by IPA as an upstream regulator of transcriptional changes occurring in response to the MH diet, which supports previous findings that PPARD is an important regulator of rumen epithelial development (Naeem et al., 2012). Of note are the relationships among ESRRA, PPAR, and members of the peroxisome proliferator-activated receptor-gamma coactivator-1 (**PGC-1**) family of transcriptional co-activators in the control of energy metabolism. First, it has been demonstrated that ESRRA directly activates *PPARA* gene expression in muscle and regulates FA metabolism and mitochondrial respiration (Huss et al., 2004). In addition, PGC-1α and PGC-1β are important regulators of ESSRA and its activation of enzymes essential for FA β-oxidation (Schreiber et al., 2003; Shao et al., 2010). Last, multiple nuclear receptors, including PPARA and PPAR-γ, are known regulators of PGC-1 (Schreiber et al., 2003). We previously identified PPARA as a transcriptional regulator in calf rumen epithelium, potentially activated by VFA, which may be important for ketogenesis and FA β-oxidation to support rumen papillary development

and differentiation during weaning (Connor et al., 2013). Likewise, PPARD was suggested as a mediator of FA metabolism and ketogenesis during rumen epithelial development (Naeem et al., 2012), and the mRNA expression of all 3 PPAR isotypes (α, δ, and γ) was detected in rumen of calves, wherein the *PPARD* and *PPARG* isotypes were most abundant (Bionaz et al., 2013). Thus, the current work suggests that activated ESRRA is an important regulator of FA absorption and metabolism in the developing rumen and may further activate PPARA to regulate ketogenesis and FA oxidation. Furthermore, multiple PPAR may indirectly affect the actions of ESRRA via their interaction with PGC-1. Therefore, due to the critical roles of VFA in rumen development and as an essential energy source for ruminants, additional evaluation of ESRRA expression and its functional interaction with PPAR and PGC-1 provide exciting areas of future investigation in ruminant physiology and nutrition.

CONCLUSIONS

New insights into the molecular mechanisms regulating rumen development and rumen epithelial cell function can be gleaned from global transcriptomic studies. Results of the present work indicate that grain feeding at weaning activates molecular pathways in rumen epithelium primarily related to the cell cycle, which appear to be regulated by transcription factors, including FOXO1 and TGFB1. Hay feeding at weaning activates gene pathways participating in energy production, in which ESRRA, likely in conjunction with PPAR, may play a prominent role in FA absorption and metabolism. Additional evidence is provided for the importance of IGF-1 and insulin as mediators of rumen epithelial cell proliferation and growth. Future investigations should focus on the interactions among VFA, ESRRA, and PPAR in FA absorption and metabolism of ruminants.

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